

Survival and humoral antibody response of Atlantic salmon, *Salmo salar* L., vaccinated against *Aeromonas salmonicida* ssp. *achromogenes*

B K Gudmundsdóttir, H Jónsdóttir, V Steinthórsdóttir, B Magnadóttir and S Gudmundsdóttir

Institute for Experimental Pathology, University of Iceland, Keldur, Reykjavík, Iceland

Abstract

Atlantic salmon were vaccinated against *Aeromonas salmonicida* ssp. *achromogenes* (Asa) by injection with three vaccines developed in our laboratory and an autogenous bacterin (IcelandBiojec.OO, IBOO) produced by a commercial vaccine producer. The humoral antibody responses to bacterial antigens were monitored by ELISA and Western blotting. The fish were challenged by infection with Asa 6 and 12 weeks post-vaccination. Protection was induced in all groups of vaccinated fish. The protection achieved was time-dependent. The autogenous bacterin, IBOO, induced a protective immune response later than our experimental vaccines. All the vaccines tested induced specific antibody response that increased between 6 and 12 weeks after vaccination. The antibody response was mainly directed against the A-layer protein, but antibodies to other bacterial components were also detected. Significant correlation was obtained between the antibody titre to extracellular Asa antigens, induced by the different vaccine preparations, and survival of vaccinated fish challenged by a virulent Asa strain. Furthermore, the detection of antibodies directed against an extracellular toxic metallo-caseinase, AsaP1, in fish sera correlated with protection.

Introduction

The fish-pathogenic bacterium *Aeromonas salmonicida* is classified into four subspecies accord-

ing to *Bergey's Manual of Determinative Bacteriology* (Holt, Krieg, Sneath, Staley & Williams 1994): ssp. *salmonicida*, ssp. *achromogenes*, ssp. *masoucida* and ssp. *smithia*. The causative agent of furunculosis in salmonid fish is *A. salmonicida* ssp. *salmonicida*, with the strains being referred to as typical. Strains of the other subspecies are termed atypical.

In Iceland, atypical furunculosis caused by *A. salmonicida* ssp. *achromogenes* is an endemic disease causing high mortality on fish farms using brackish water for rearing salmonid fish and in captive cod of wild origin cultivated in sea cages. Furthermore, the susceptibility of halibut to the bacterium has been shown by experimental infection. The bacterium has been isolated from wild marine fish species and clinical signs are frequently detected in infected wild salmonids. The disease is systemic and external signs are similar to furunculosis including haemorrhages and lesions (furuncles) in skin and muscle (Gudmundsdóttir, Gudmundsdóttir, Magnadóttir & Helgason 1996).

Typical and atypical *A. salmonicida* strains have been reported to share cell-associated antigens such as the A-layer protein, the LPS component, iron-regulated outer membrane proteins and porins (Evenberg, Verslius & Lugtenberg 1985; Pyle & Cipriano 1986; Chu, Cavaignac, Feutrier, Phipps, Kostrzynska, Kay & Trust 1991; Hirst & Ellis 1994; Lutwyche, Exner, Hancock & Trust 1995). On the other hand, exotoxins produced by atypical strains, including ssp. *achromogenes*, differ from those of typical strains (Ellis 1991; Austin & Austin 1993; Gudmundsdóttir 1996).

The majority of the literature on vaccination and

Correspondence Bjarnheidur K. Gudmundsdóttir, Institute for Experimental Pathology, University of Iceland, Keldur, v/Vesturlandsveg, IS-112, Reykjavík, Iceland

immunity of *A. salmonicida* reports studies dealing with typical strains and salmonids. Furthermore, commercially available *A. salmonicida* vaccines are produced from typical *A. salmonicida* strains for prevention of furunculosis in salmonids. Diseases caused by atypical strains are of increasing importance world-wide and the prospects for their control by vaccination need to be considered. The aim of the present study was to evaluate the efficacy of three vaccines developed in our laboratory and one autogenous bacterin produced by a commercial vaccine producer in protection of Atlantic salmon, *Salmo salar* L., against a challenge with *A. salmonicida* ssp. *achromogenes*, and to correlate the humoral immunity induced with the levels of protection observed.

Materials and methods

Fish

Atlantic salmon fingerlings with an average weight of 25 g, which were the offspring of wild fish from an Icelandic river, were used in the study. The fish came from a fish hatchery (Laxeldisstöðin Kollafirði) where atypical furunculosis had never been detected. During injection and bleeding procedures, the fish were anaesthetized by immersion in benzocaine at a concentration of 40 mg l⁻¹. Before vaccination, the fish were marked with an Alcian blue dye and acclimatized for one week. The fish were kept in aquaria at 10 ± 3°C with continuously flowing (1 l min⁻¹) well water and fed commercial dry pellets (Vextra mini 1.6 mm, EWOS), 2% body weight per day, with an automatic feeder.

Bacteria

Three *A. salmonicida* ssp. *achromogenes* isolates (265–87, M108–91 and S24–92), originating from diseased Atlantic salmon from three fish farms in Iceland, were used in the study. The isolates were homogeneous with respect to biochemical reactions, antibiograms and virulence properties, and showed the following characteristics: Gram-negative, non-motile, facultatively anaerobic, short rods; production of indole and acid from sucrose; failure to produce gas from glucose and to degrade aesculine; resistance to the antibiotics ampicillin and cephalothin; autoaggregation and delayed production of brown pigment. All three isolates possessed two well-known cell-associated virulence

factors, LPS (lipopolysaccharide) and the A-layer, and secreted proteases comparable with those of the type strain, NCMB 1110, for ssp. *achromogenes*, including a toxic caseinolytic metallo-protease, AsaP1 (Gudmundsdóttir 1996).

Vaccines

Experimental vaccines were made by cultivation of isolate 265–87 in brain heart infusion broth, BHIB (BHI, Oxoid, Lyfjaverslun Íslands, Iceland) containing 100 µg ml⁻¹ of the iron chelator ethylenediamine di(o-hydroxyphenyl-acetic acid) (EDDA) for 72 h at 22°C in a laboratory fermenter (KLF 2000, Bioengineering, Wald, Switzerland). After cultivation, the bacteria were killed by incubation in 2% (w/v) formaldehyde for 24 h at 22°C. Cells were separated from the extracellular products (265–87-ECP) by centrifugation (6000 g for 30 min) and resuspended in phosphate buffered saline, PBS (pH 7.2). The absorbance of the cell suspension measured at 600 nm against a substrate blank was adjusted to 0.8 (OD₆₀₀). For further inactivation of the toxic enzyme activity, the ECP was treated either by dialysing overnight in 25 volumes of 10 mM OPA (1, 10-phenanthroline), 1 mM PMSF in PBS for 4 h at 22°C (chemical treatment), heating for 15 min at 55°C (heating) or both. Following inactivation, the ECP was concentrated by dialysis in polyethylene glycol at 4°C to approximately one-fourth of its original volume. Protein concentration of ECPs was determined by the Bradford protein assay (BioRad, Lyfjaverslun Íslands, Iceland) and adjusted to 200 µg ml⁻¹ by dilution in PBS. The experimental vaccines were composed of 10 volumes of ECP mixed with one volume of cell-suspension emulsified in an equal volume of Freund's incomplete adjuvant (FIA).

Three experimental vaccines produced from strain 265–87 were used in the study: K-14, containing ECP inactivated by heating; K-15, containing ECP inactivated by chemical treatment; and K-16, containing ECP inactivated by chemical treatment and heating. An autogenous bacterin, Iceland Biojec.OO (IBOO), containing a mineral oil adjuvant, produced by a commercial vaccine producer (Alpharma N.W. Inc., Bellevue, WA, USA) from strain M-108–91 was also included in the study.

Two groups served as control: one was injected with 0.1 ml PBS and the other with 0.1 ml PBS emulsified in FIA.

Table 1 Experimental protocol*

Total number of fish	Injected with:	Dose (ml)	Number sampled		Number challenged		
			6 weeks p.v.	12 weeks p.v.	Challenge dose: 5000 CFUs		Challenge dose: 1000 CFUs:
					6 weeks p.v.	12 weeks p.v.	
130	PBS	0.1	25	15	30	30	30
120	FIA	0.1	15	15	30	30	30
65	K-14	0.1	0	5	0	30	30
138	K-15	0.1	24	24	30	30	30
65	K-16	0.1	0	5	0	30	30
129	IBOO	0.2	21	18	30	30	30

*Key: CFUs, colony forming units; p.v., post-vaccination; FIA, Freund's incomplete adjuvant; K-14, K-15 and K-16, experimental vaccines developed at our laboratory; IBOO, an autogenous bacterin produced by A.L. Pharma Inc.

Immunization and challenge

A total of 647 Atlantic salmon fingerlings were included in the study. The vaccines, as well as the control injections of PBS or FIA, were administered intraperitoneally (i.p.). A dose of 0.1 ml was used for each vaccine, except for 0.2 ml of IBOO injected according to the producers instructions. The detailed immunization protocol is shown in Table 1.

Challenges were performed 6 and 12 weeks post-vaccination by intramuscular injection (i.m.) of isolate S24-92, according to the protocol given in Table 1. Deaths were recorded daily for 4 weeks. The cause of mortality was established by reisolation of *A. salmonicida* ssp. *achromogenes* from the head kidney.

Sampling

Caudal blood samples were taken 6 and 12 weeks post-vaccination according to the procedure described in Table 1. Blood samples were allowed to clot at 15°C for 4 h and 4°C overnight, centrifuged at 2000 g for 10 min, and the serum sampled. Samples from five fish in each group were pooled (sera pools) and the remaining serum samples from individual fish (10–20) were kept separate. Sera were stored at –20°C until used for antibody screening.

Antigen preparations

The ECP-antigen, 265–87-ECP, was concentrated by dialysis in polyethylene glycol at 4°C to approximately one-fourth of its original volume and desalted on a Sephadex G-25M (Pharmacia PD10) column. Protein concentration of ECPs was measured with the Bradford protein assay and adjusted to 600 µg ml⁻¹ by dilution in PBS.

The ECP-cell-antigen, 265–87-cell suspension (OD₆₀₀ = 0.8), was sonicated for 5 min at an amplitude of 14–18 µm. Ten volumes of ECP-antigen were mixed with one volume of sonicated cell suspension.

Double sandwich anti-ECP ELISA

The assay has been described in detail elsewhere (Magnadóttir & Gudmundsdóttir 1992). Briefly, ELISA trays (Nunc) were coated overnight with 100 µl per well of either ECP- or ECP-cell antigen (10 µg protein ml⁻¹) diluted in ELISA coating buffer (0.1 M sodium carbonate buffer, pH 9.6), blocked with BSA and overlaid with 100 µl of test sera in two-fold serial dilutions in PBS containing 0.05% Tween-20 (PBS-T). After an overnight incubation at 4°C, bound antibody was detected with a polyclonal mouse antibody to salmonid IgM (raised against Atlantic salmon IgM at our laboratory), followed by peroxidase labelled rabbit antibody to mouse immunoglobulins (Dako). Incubations were performed for one hour at 37°C. Colour was developed with 1,2-phenylenediamine dihydrochloride (OPD) substrate (Sigma) for 30 min at 22°C. Between each step, wells were washed extensively with PBS-T. Optical density (OD) was read at 492 nm (OD₄₉₂). The serum titre was determined as the reciprocal value of the highest dilution that gave an OD reading above 0.2 at 492 nm against a negative control blank where the serum was replaced by PBS-T.

Western blotting

The antigen preparations (ECP- and ECP-cell-antigen) were incubated for 5 min at 100°C with

sample buffer (1/1 v/v) comprised of 8% SDS and 10% 2-mercaptoethanol in Tris-HCl, pH 6.8. Separation of proteins was performed by SDS-PAGE, using 14% resolving polyacrylamide gels with 4.5% stacking gel and the Mini-Protean II system from BioRad according to the manufacturer's instructions. Each gel was overlaid with 300 µl of antigen solution containing 330 µg protein. Molecular weight markers were BioRad, LMW.

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane by electrophoresis at 100 V and 250 mA for 90 min. The buffer used for transfer was 25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.8. Following transfer, the membranes were either stained for total proteins with colloidal gold (AuroDye, Amersham, England) or blocked (1 h at 22°C) with 1% BSA and 1% normal swine serum in PBS-T. Primary and secondary antibodies as well as the alkaline phosphatase labelled conjugated antibodies (from BioRad) were diluted in PBS-T containing 0.2% BSA and 0.2% swine serum. Incubation with primary antibody was at 4°C overnight, other antibodies were incubated for 1 h at room temperature. The blots were washed extensively in PBS-T between each step.

For detection of salmon antibodies, the membranes were probed with immune or normal fish serum diluted 1/50, followed by incubation with a secondary polyclonal mouse antibody to salmon IgM (prepared at our laboratory) diluted 1:2000.

For detection of *A. salmonicida* A-layer protein and the o-polysaccharide component of LPS, the membranes were probed with murine monoclonal antibodies diluted 1:1000. These were kindly provided by S. Esplid, FORUT, Tromsø, Norway.

Bound antibody was detected with AP-labelled rabbit antibody to mouse immunoglobulins (Dako, Lyfjaverslun Íslands, Iceland). Immunoreactive bands were visualized by incubating membranes in buffer containing: 0.1 M Tris-HCl, pH 8.0; 0.1 M NaCl; 0.05 M MgCl₂; 0.33 mg ml⁻¹ p-nitro blue tetrazolium chloride (NBT); and 0.175 mg ml⁻¹ 5-bromo-4-chloro-3 indolyl phosphate toluidine salt (BCIP).

Statistical analysis

Mortalities and anti-ECP antibody titres were compared between groups with a chi-square test. Survival in different groups were compared with serum titres by Spearman rank correlation.

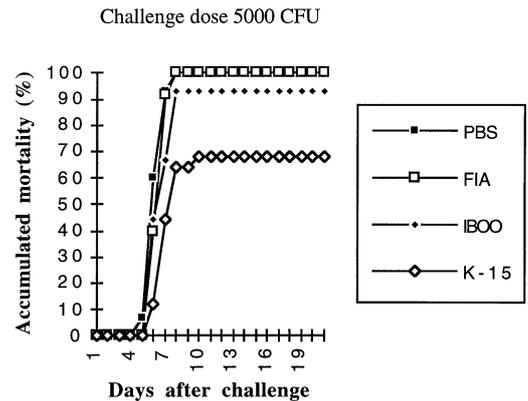


Figure 1 Challenge 6 weeks after control injection or vaccination. Accumulated mortality (%) of Atlantic salmon for 21 days after an intramuscular challenge with 5000 CFUs of *A. salmonicida* ssp. *achromogenes*, strain S24–92: phosphate-buffered saline (PBS) and Freund's incomplete adjuvant (FIA), control groups; K-15 experimental vaccine developed at our laboratory; IBOO, autogenous bacterin produced by A. L. Pharma Inc.

Results

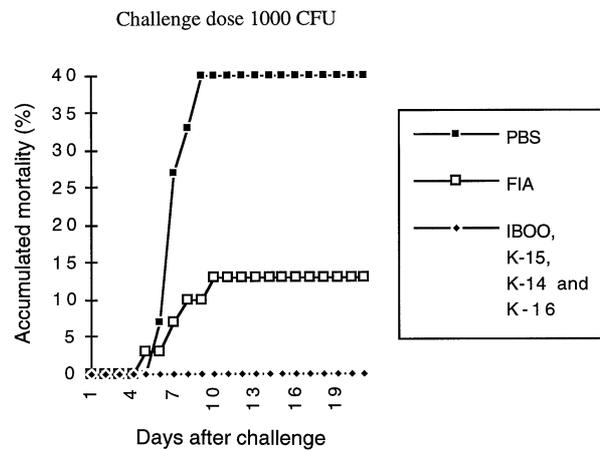
Challenges

Accumulated mortalities in groups of Atlantic salmon challenged 6 and 12 weeks after vaccination or control injection of PBS or FIA are illustrated in Figs 1 and 2. The *A. salmonicida* ssp. *achromogenes* (Asa) strain (S24–92) used for challenge was highly virulent, causing 40% and 100% mortality in the PBS control groups at challenge doses of 1000 and 5000 colony-forming units (CFUs) per fish, respectively.

No fish in the control groups survived a challenge with 5000 CFUs of Asa at 6 weeks. At 12 weeks, 4% of the FIA injected controls but none of the PBS controls survived (Tables 2 & 3). The difference between the controls challenged with the higher dose at 12 weeks was not significant ($P = 1.0000$). On the other hand, a significantly higher survival was observed in the FIA controls than in the PBS group following challenge at 12 weeks with a lower dose, 1000 CFUs, $P = 0.0391$ (Table 3).

Survival in groups treated with experimental vaccines (K-14, K-15 and K-16) was significantly higher ($P \leq 0.004$) than in the control injected fish (PBS and FIA) at both 6 and 12 weeks. Survival of the IBOO-vaccinated fish was not significantly higher than the controls ($P = 0.4910$) 6 weeks after vaccination, but it was significant 12 weeks after vaccination ($P < 0.0001$) (Tables 2 & 3). Survival of the salmon immunized with chemically inactivated vaccine K-15, and challenged with 5000 CFUs Asa

A



B

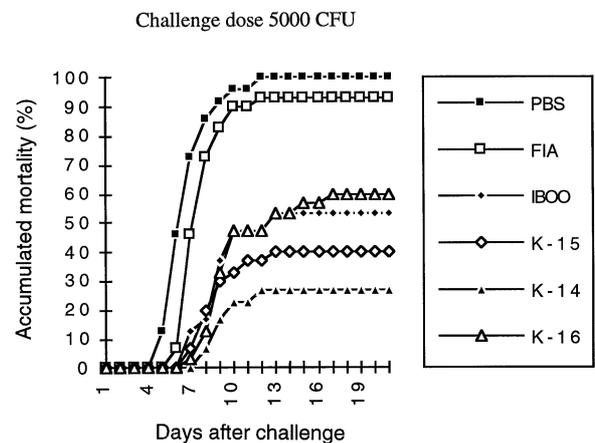


Figure 2 Challenge 12 weeks after control injection or vaccination. Accumulated mortality (%) of Atlantic salmon for 21 days after an intramuscular challenge with (A) 1000 or (B) 5000 CFUs of *A. salmonicida* ssp. *achromogenes*, strain S24-92: phosphate-buffered saline (PBS) and Freund's incomplete adjuvant (FIA), control groups; K-14, K-15 and K-16, experimental vaccines; IBOO, autogenous bacterin produced by A. L. Pharma Inc.

Table 2 Antibody titre against *Aeromonas salmonicida* ssp. *achromogenes* ECP-antigen in sera pools measured with ELISA and survival of Atlantic salmon challenged 6 weeks after vaccination

Treatment group	Antibody titre of sera pools ($n = 5$)	Challenge dose: 5000 CFUs* per fish	
		Survival (%) ($n = 30$)	χ^2 (d.f. = 1) [†] P value
PBS (negative control)	<100	0	–
FIA (adjuvant control)	<100	0	–
K-15 (experimental vaccine)	1600	38	0.0040
IBOO (autogenous bacterin)	400	7	0.4910

*CFUs, colony forming units.

[†]For comparison with mortality in the PBS-control group.

increased from 38% at 6 weeks to 60% at 12 weeks, but the difference was not statistically significant ($P = 0.0692$). Corresponding values for the groups immunized with the IBOO vaccine were 7% and

48%, respectively. This shows that the IBOO vaccine was more effective at the later challenge date ($P = 0.0005$).

Comparison of the effect of various vaccine

Table 3 Antibody titre measured with ELISA against *Aeromonas salmonicida* ssp. *achromogenes* ECP-antigen in sera samples and survival of Atlantic salmon challenged 12 weeks after vaccination

Treatment group	Antibody titre of sera pools ($n = 5$)	Challenge dose: 1000 CFUs* per fish		Challenge dose: 5000 CFUs per fish	
		Survival (%) ($n = 30$)	χ^2 (d.f. = 1) [†] P value	Survival (%) ($n = 30$)	χ^2 (d.f. = 1) [†] P value
PBS (negative control)	<100	60	–	0	–
FIA (adjuvant control)	<100	86	0.0391	4	1.0000
K-14 (experimental vaccine)	102 400	100	<0.0001	73	<0.0001
K-15 (experimental vaccine)	102 400	100	<0.0001	60	<0.0001
K-16 (experimental vaccine)	25 600	100	<0.0001	40	0.0001
IBOO (autogenous bacterin)	1600	100	<0.0001	48	<0.0001

*CFUs, colony forming units.

[†]For comparison with mortality in the PBS-control group.

preparations at 6 weeks showed that a significantly better ($P = 0.0109$) protection against an Asa (5000 CFUs) challenge was achieved with the K-15 vaccine than the IBOO vaccine (Tables 2 & 3). In the higher challenge (5000 CFUs) at 12 weeks, the protective effect of K-14 (heat inactivated) was significantly better ($P = 0.0182$) than that of K-16 (heat and chemically inactivated), but the difference between the other vaccines was not statistically significant ($P > 0.05$).

As shown in Table 3, all vaccinated salmon challenged with the lower dose, 1000 CFUs Asa, at 12 weeks survived, as did 86% of the FIA controls and 60% of the PBS controls. The difference in survival of vaccinated fish and FIA injected fish, respectively, was not significant ($P = 0.0541$), whereas comparison of the PBS controls with both the FIA controls and the vaccinated groups showed a significant difference ($P < 0.04$).

Detection of anti-ECP antibodies in sera from immunized Atlantic salmon by ELISA

The antibody response to the ECP-antigen measured by ELISA 6 and 12 weeks post-vaccination is shown in Fig. 3, and Tables 2 and 3.

The antibody titre of pooled samples 6 weeks post-vaccination was four times higher in fish vaccinated with the experimental vaccine K-15 than with the autogenous bacterin, IBOO (Table 2). As shown in Table 3, the response continued to increase for the remaining 6 weeks of the experiment, resulting in a 64-fold increase in antibody titres of the K-15 sera pool and a four-fold increase of the IBOO sera pool. The antibody responses in sera from K-14- and K-16-vaccinated fish were only measured in pools of sera ($n = 5$) sampled at 12 weeks. At this time, the

K-14 and K-15 injected fish had the same antibody titres, but the titre of the K-16 injected fish was twice as low (Table 3).

An analysis of the specific antibody response of individual fish at 6 and 12 weeks post-vaccination, from PBS and FIA controls, and K-15- and IBOO-vaccinated fish is shown in Fig. 3. An anti-ECP titre higher than 100 was detected in 2.5% of the PBS controls and 16.7% of the FIA controls when both samples taken at 6 and 12 weeks were included. The difference between the titres of PBS and FIA controls was not significant ($P = 0.1394$), but the difference in titres of vaccinated and control injected fish was highly significant ($P < 0.0001$). Significantly lower titres were obtained in the IBOO than in the K-15-vaccinated fish at both 6 ($P = 0.045$) and 12 weeks ($P = 0.0032$). Thus, 73% of the K-15-treated fish and 19% of the IBOO-treated fish had a titre higher than 800 after 6 weeks, and the corresponding values after 12 weeks were 95% and 38%, respectively.

Analysis of anti-ECP and anti-ECP-cell antibodies by immunoblotting

The antisera pools sampled 6 and 12 weeks after vaccination were used for immunostaining of Western blots of extracellular products (ECP-antigen), and a mixture of ECP and cell suspension (ECP-cell-antigen) were employed to analyse the antigens stimulating antibody production in immunized Atlantic salmon. Western blotting showed that antibody response in all groups of vaccinated fish were mainly directed towards the *A. salmonicida* A-layer protein (Figs 4 & 5).

Figure 4A shows ECP-antigen immunostained with sera sampled 6 weeks after vaccination. No bands were clearly visualized by PBS and FIA control sera.

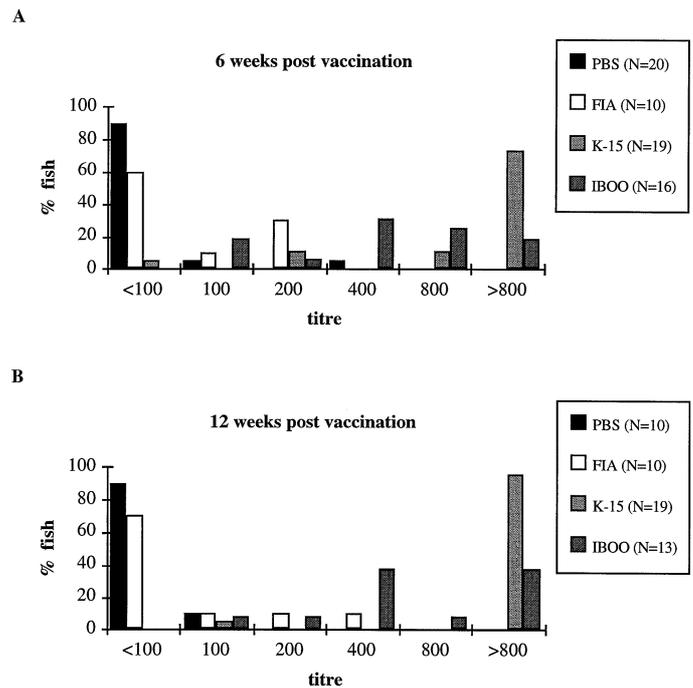


Figure 3 Antibody titre measured with ELISA against *Aeromonas salmonicida* ssp. *achromogenes* ECP-antigen in sera sampled from fish in each treatment group (A) 6 and (B) 12 weeks post-vaccination.

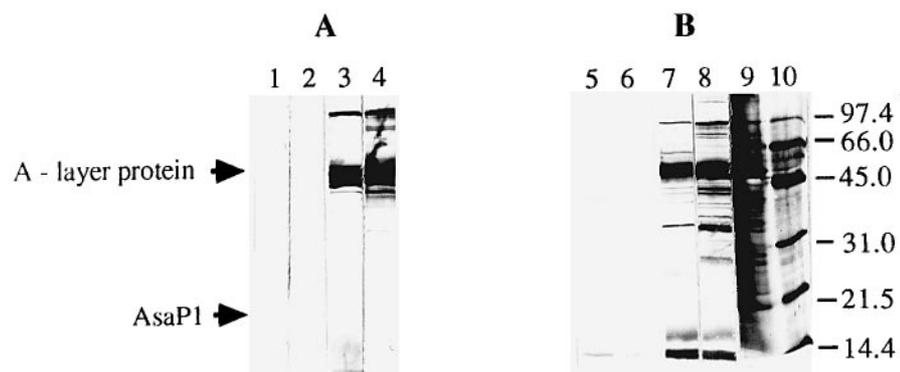


Figure 4 Western blots of *A. salmonicida* ssp. *achromogenes*, strain 265–87, (A) ECP-antigen or (B) ECP-cell-antigen. Proteins detected by immunostaining with sera pools ($n = 5$) diluted 1:50 sampled from Atlantic salmon 6 weeks after treatment: lanes 1 and 5, PBS (control); lanes 2 and 6, FIA (adjuvant control); lanes 3 and 7, K-15 (experimental vaccine); and lanes 4 and 8, IBOO (autogenous bacterin). Proteins detected by colloidal gold staining: lane 9, ECP-cell-antigen; and lane 10, molecular weight standards (kDa).

In addition to the A-layer protein, the sera from K-15- and IBOO-treated fish strongly stained a component with a molecular weight (MW) above 100 kDa. Furthermore, a component with MW of about 43 kDa was stained with both these sera. A 20-kDa component was weakly stained with sera from K-15-treated fish. Sera from the IBOO-treated group also stained several components with different MWs.

Figure 4B shows ECP-cell-antigen immunostained with sera sampled 6 weeks after vaccination. More bands were visualized with sera from the IBOO-

than the K-15-treated fish in this antigen, as with the ECP-antigen. All components stained by sera of the K-15-treated group were also stained by the anti-IBOO sera. A 20-kDa band was not detected in this antigen preparation by any sera.

Figure 5A shows ECP-antigen immunostained with sera sampled 12 weeks after vaccination. The anti-IBOO sera stained at least four components besides the A-layer protein, including the 20-kDa component (a caseinase detected by overlaying a gel with casein), which were also stained by sera from all

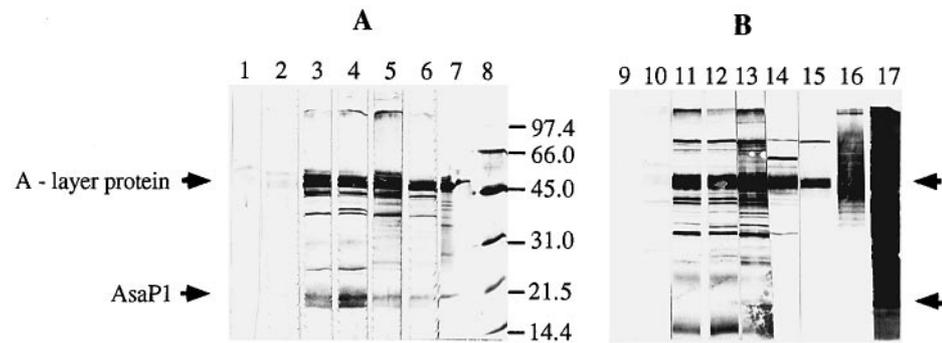


Figure 5 Western blots of *A. salmonicida* ssp. *achromogenes*, strain 265–87, (A) ECP-antigen or (B) ECP-cell-antigen. Proteins detected by immunostaining with sera pools ($n = 5$) diluted 1:50 sampled from Atlantic salmon 12 weeks after treatment: lanes 1 and 9, PBS (control); lanes 2 and 10, FIA (adjuvant control); lanes 3 and 11, K-14; lanes 4 and 12, K-15; lanes 5 and 13, K-16; and lanes 6 and 14, IBOO. Proteins detected by colloidal gold staining: lane 7, ECP-antigen; lane 8, molecular weight standards (kDa); and lane 17, ECP-cell-antigen. Proteins detected by immunostaining with monoclonal antibodies: lane 15, A-layer protein; and lane 16, LPS o-antigen.

other vaccinated groups. Only sera from the K-14- and K-15-injected groups clearly stained a component with a MW of around 25 kDa, and the anti-K-15 sera stained one band (MW close to 40 kDa) which was not visualized by any other sera.

Figure 5B shows ECP-cell-antigen immunostained with sera sampled 12 weeks after vaccination. The anti-IBOO sera stained at least four different components, and the sera from fish immunized with the experimental vaccines (K-14, K-15 and K-16) stained at least 10 components each. Sera from salmon vaccinated with all three experimental vaccines showed similar staining patterns. All bands stained with anti-IBOO sera, except a component with MW close to 60 kDa, were also stained by sera derived from salmon in the three other vaccinated groups.

Survival compared with antibody titre

Correlation between the antibody titre to the ECP antigens detected by ELISA and survival of salmon in challenge experiments at 6 (Table 2) and 12 weeks (Table 3) was highly significant ($P = 0.0003$) as determined by Spearman rank correlation.

Discussion

The results presented in this paper show that Atlantic salmon can be successfully vaccinated against infection of *Aeromonas salmonicida* ssp. *achromogenes* (Asa) by injection of bacterins containing a mineral oil adjuvant. A significantly lower mortality was achieved in all vaccinated groups than both PBS and adjuvant (FIA) controls, when the salmon were

challenged with a dose containing 5000 CFUs of a highly virulent Asa strain (S24–92). However, if challenged with a lower dose (1000 CFUs), the difference in protection of vaccinated fish and the FIA controls was not quite significant, but survival of the FIA controls was significantly higher than that of the PBS controls. Apparently, the FIA had some protective effect against the lower challenge dose, which might be caused by stimulation of unspecific mechanisms and/or by stimulation of cellular immune response. Our earlier studies have shown that FIA has a stimulatory effect on the proliferation of leucocytes in Atlantic salmon (Gudmundsdóttir, Magnadóttir & Gudmundsdóttir 1995). This is also in accordance with reports on the importance of FIA-bacterin combinations in provoking a protective immune response to infection with typical *A. salmonicida* in Atlantic salmon (Cipriano & Pyle 1985; Anderson 1992). The protective effect of the different vaccine preparations against the higher dose challenges showed some variation. The K-15 vaccine, containing chemically inactivated ECP, was significantly more effective than the autogenous bacterin, IBOO, in a challenge performed 6 weeks post-vaccination. On the other hand, the only significant difference obtained in challenges at 12 weeks was that the K-14 vaccine, containing heat-inactivated ECP, was more efficient than the K-16 vaccine, which contains ECP inactivated by chemicals and heat. The protection achieved was time-dependent because an improvement was observed when the fish were challenged 12 weeks after vaccination as compared with 6 weeks.

Analysis by ELISA of anti-ECP antibody response

to the different vaccine regimes showed that all induced specific antibodies that increased in anti-ECP titre from weeks 6 to 12. The salmon vaccinated with the experimental vaccines (K-14, K-15 and K-16) had significantly higher anti-ECP titres than the one vaccinated by the IBOO vaccine. Both the time course and the antibody response of individual fish showed some variation. Survival of salmon in Asa challenges correlated significantly with anti-ECP titres, indicating the protective nature of the humoral immune response.

The specific antibodies were mainly directed against the A-layer protein. However, their protective role appeared questionable. Thus, the IBOO vaccine induced a stronger antibody response against the A-layer than K-15 at 6 weeks, as demonstrated in Western blots (Fig. 4). The challenge test performed at that time revealed that the IBOO vaccine did not have a significant effect on survival, whereas the K-15 vaccine did. This is in accordance with our previous findings showing that rainbow trout antisera containing high amounts of antibodies to the A-layer protein were not protective for passively immunized Atlantic salmon (Gudmundsdóttir & Magnadóttir 1997). In another recent study (Magnadóttir, Gudmundsdóttir & Gudmundsdóttir 1995), a high response directed against the A-layer protein and LPS (o-antigen) was found in diseased Atlantic salmon naturally infected by Asa. The response apparently had limited protective value as the disease progressed quickly unless controlled by other means, such as antibiotics. Recent studies by Killie & Jørgensen (1994, 1995) explore the importance of antigen-induced suppression in immunoregulation in Atlantic salmon. In this respect, the dominating antigenicity of the A-layer protein could possibly establish a suppression of humoral immunity against protective antigens, which would explain the low protection induced by IBOO 6 weeks post-vaccination. In the present study, detection of antibodies against the toxic metallo-caseinase, AsaP1, correlated with protection. Thus, the protective effect of the IBOO bacterin in challenges at 12 weeks correlated with the appearance of anti-AsaP1 antibodies. Furthermore, the survival in the K-14 group was significantly higher than in the group vaccinated with K-16 and the main difference in the antibody response obtained was a stronger staining of AsaP1 in Western blots by sera from the K-14 immunized group. The significance of anti-AsaP1 antibodies in protection of Atlantic salmon against an Asa infection has been reported in a previous study

(Gudmundsdóttir & Magnadóttir 1997). Passive immunization of Atlantic salmon with rainbow trout anti-AsaP1 antisera was shown to confer significant protection against an Asa challenge. In the same study, Asa ECPs were found to elicit better protection than whole bacteria in actively immunized Atlantic salmon and the protection strongly correlated with the detection of antibodies directed against AsaP1 in fish sera. In the present study, production of antibodies against several proteins, other than the two mentioned above, were induced by the different vaccine preparations. All sera pools obtained from fish vaccinated with experimental vaccines K-14, K-15 and K-16 stained similar bands. At 6 weeks post-vaccination, more bands were stained by the anti-IBOO than the anti-K-15 sera in both antigens tested. The protection induced by IBOO by that time was very limited, so the significance of these antigens in protection is questionable. Furthermore, the anti-IBOO sera stained fewer bands than the other sera pools from vaccinated salmon at 12 weeks. One strong band with a MW close to 60 kDa was detected in the ECP-cell-antigen by the anti-IBOO sera taken at 12 weeks, but not by any other sera. The nature of this antigen is unknown. AsaP1 was only detected in the ECP-antigen, although a 20-kDa component was also clearly visible in the ECP-cell-antigen. This remains unexplained.

The results obtained in this study do not elucidate the relationship between the nature of the antigens and protection. Non-specific defence mechanisms as well as cellular immune response may play some role in the protection obtained in the vaccinated salmon. However, the good correlation observed between anti-ECP-antibody titre and survival of vaccinated fish supports previous findings describing the importance of humoral antibody response and neutralization of bacterial toxins in protecting Atlantic salmon against *A. salmonicida* ssp. *achromogenes* (Gudmundsdóttir & Magnadóttir 1997).

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